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ABSTRACT

The transforming growth factor β (TGF-β) signaling pathway is an essential pathway that results in cell growth arrest when initiated in most epithelial cells. Initiation of TGF-β receptors leads to the phosphorylation and translocation of the Smad proteins, the major TGF-β intracellular signaling molecule, to the nucleus where transcription of TGF-β target genes occur. Aberrations in the Smad proteins are present in many breast cancers indicating their importance. Understanding how post-translational modification such as phosphorylation and acetylation regulate pathways will increase our knowledge of how a normal cell becomes cancerous and may provide insight into novel therapeutics.

This proposal suggests a series of experiments designed to study whether acetylation of Smad proteins occurs. Our lab has determined that Smad2, but not Smad3, can be acetylated by the acetyltransferase protein p300 in vivo and in vitro. The residues required for acetylation have been mapped and mutated resulting in a Smad2 mutant that cannot be acetylated. This mutant still retains its ability to become phosphorylated and bind Smad4 without affecting its stability. However, preliminary studies suggest a larger cytoplasmic fraction of the mutant Smad2 upon TGF-β treatment in comparison to the wild type Smad2 protein.

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Introduction

Initiation of the TGF-β signaling pathway results in a wide array of biological functions in different cell types. A few biological functions affected by TGF-β signaling include cell growth, differentiation, migration, and apoptosis [1-3]. The importance of the TGF-β signaling pathway is evident by the number of human diseases, including breast cancer, that have mutations in different components of this pathway [4]. Mice models indicate TGF-β initially works to prevent tumor progression [5]. However, the role TGF-β plays in cancer progression is very complex as shown by evidence indicating that TGF-β can promote tumor progression in advanced breast cancers [6]. Thus, understanding the dual role of TGF-β in the initiation and progression of tumors will provide essential knowledge on how cancers arise and progress.

TGF-β signaling is initiated when TGF-β binds to and activates the TGF-β receptor serine/threonine kinase complex [7-8]. The activated complex phosphorylates the c-terminal of Smad2 and Smad3, allowing oligomerization with the common mediator Smad4 [9-10]. The Smad2/3/4 complex then translocates to the nucleus where it recruits coactivators like p300/CBP to initiate transcription of TGF-β target genes [11-13].

The coactivators p300 and CBP were first identified as E1A associated and CREB binding proteins [14-15]. While they can act as scaffolding and bridging proteins, the most studied function of these two proteins is its ability to acetylate histones, a hallmark of transcriptionally active chromatin [16-17]. In recent years, many non-histone proteins such as p53, β-catenin, and the Smad7 have been accepted as acetylated proteins [18-20]. The modification of these proteins with an acetyl group has a variety of consequences on the biochemical properties of each protein. Some consequences include altered protein stability, subcellular localization, and protein interactions [21-22]. Acetylation can also compete with other lysine modifications such as ubiquitination and sumoylation. For example, Smad 7, an inhibitory Smad that suppresses TGF-β signaling, competes with ubiquitination by modifying common lysines, protecting it from proteosomal degradation [20]. Acetylation of Smad7 decreases upon stimulation with TGF-β.

The c-termini of Smad1, 2, 3, and 4 have been shown to directly interact with the c-terminus of p300/CBP in a ligand dependent manner [12,13,23,24]. This interaction increases the transcriptional activity of the Smad proteins. However, not much else is known about the role of this interaction in TGF-β signaling. We were initially interested in whether the recruitment of p300/CBP to the Smad complex could result in acetylation of any of the Smad proteins and if so, what the role acetylation played in the regulation of TGF-β. Understanding this modification would offer knowledge of a new layer of regulation for the TGF-β signaling that is likely perturbed in beast cancer, thus possibly arising in new ideas for the treatment or early detection of breast cancer.

Body

As of last year, we had determined that Smad2, but not Smad3, could be acetylated in a p300 dependent manner. Both in vivo and in vitro data support the conclusion that only Smad2 could be acetylated by p300. This result is particularly interesting because Smad2 and Smad3 have 92% sequence identity. Both proteins contain a conserved MH1 domain at the N-terminus linked to a conserved C-terminal MH2 domain by an unconserved proline-rich linker. The major difference between the two proteins in the presence of two sequence inserts, named the GAG and TID region, in the MH1 domain of Smad2 that provides steric interference that prevents direct DNA binding [25]. Smad2 requires the presence of Smad4 to bind DNA while Smad3 can directly bind DNA. The acetylation of Smad2 was also independent of Smad2 phosphorylation; when Smad2 mutants where the phosphorylated serines have been mutated to alanine were still acetylated to the same extent as wild-type Smad2 when co-transfected into 293T cells with full-length p300. Thus, acetylation of Smad2 does not depend on its phosphorylation state.

Originally, we had planned on using Smad2 truncations to isolate the regions acetylation occurred in order to better determine which residues were modified. However, the Smad2 truncations proved to be unstable. Fortunately, we were able to use flag-tagged Smad1/Smad2 chimeras made by a previous graduate student to determine that the acetylation of Smad2 occurs in the MH1 domain of Smad2. Alignment analysis identified one lysine residue, lysine 144, found in the MH1 domain of Smad2 that was not present in Smad3. However, when this lysine was mutated to alanine there was no significant decrease in level of acetylation suggesting that lysine 144 is not acetylated. More Smad1/Smad2 chimeras were cloned to better isolate where acetylation occurred. Lysine residues in the regions of interested were mutated to alanine and it was determined that lysines 19 and 20 are required for acetylation of Smad2. It is still unclear whether or not these residues are regulatory residues or the actual residues being modified so mass spectrometry experiments are currently in progress to determine the exact lysines modified.

Knowing that the lysines required for acetylation resided adjacent to the GAG region caused us to wonder if this region was important for Smad2 acetylation. If so, it would explain why Smad2 but not Smad3 was acetylated in a p300 dependent manner. Smad2 mutants were made where either the GAG region (Smad2?GAG) or the TID (Smad2?TID) region was deleted. When cotransfected into 293T cells with p300, the Smad2?GAG mutant lost the ability to be acetylated while the Smad2?TID mutant was still acetylated to the same extent as the wild type. Most likely the GAG region alters the structure of Smad2 in a way that will present lysines 19 and 20 to p300 more efficiently compared to Smad3. Unfortunately, while the crystal structure of the Smad3 MH1 region has been solved, the MH1 region of Smad2 is still unknown. Currently mutant of Smad3 where the GAG region has been inserted have been made and experiments are in progress to see whether adding the GAG region will enable Smad3 to now become acetylated.

Knowing which residues are acetylated will be important for determining the consequence of Smad2 acetylation. The Smad2 non-acetylated mutant (Smad2 2K19R) is a great tool for testing different biochemical properties. When tested in parallel with the wild type Smad2, the Smad2 2K19R mutant did not have altered binding ability to known Smad2 binding partners like Smad 4 or Fast1. Likewise, the Smad2 2K19R mutant retained its ability to be phosphorylated when stimulated with TGF-ß. The Smad2 2K19R mutant also did not exhibit any altered DNA binding ability in gel shift experiments.

When doing the in vivo experiments to test whether Smad2 was acetylated when co-transfected with p300 we always observed that the acetylated Smad2 had more protein present on Western blot compared to the non-acetylated Smad2. The Smads that were not acetylated, for example Smad3, did not see as huge of an increase in the amount of protein present. Thus, the

possibility that acetylation of Smad2 affected protein stability was promising to us. The ability of acetylation to alter protein stability is well documented. Many proteins such as p53 and the TGF-ß inhibitor Smad7, are acetylated and ubiquitinated on common lysines. When the protein is acetylated it competes with ubiquination and prevents protein degradation through the proteosomal pathway. To test whether this could be true for Smad2 a pulse chase experiment was performed using the Smad2 2K19R mutant. Smad2 or Smad2 2K19R was overexpressed in 293T cells with and without the presence of p300. The protein was pulsed for 30 minutes and then chased at 0 hour, 2 hour, 4 hour, 8 hour, 12 hour, and 24 hour. Unfortunately, even at the 24 hour time-point there was no discernible decrease in labeled Smad2 or Smad2 2K19R levels. We were expecting the run into some difficulty since it is well known that Smad2 is a very stable protein but were surprised when even at 24 hour there was no decrease in protein level. It is possibly that because we are doing this experiment using overexpressed protein we are labeling such high levels of protein that we are over-saturating the system and thus masking any protein degradation that we could see. Thus, this experiment will be repeated in both stable cells lines and in cells transiently over-expressing less protein and with a shorted pulse to see whether labeling less protein will enable us to see some protein degradation.

Acetylation is also known to affect the localization of proteins, primarily through altered binding to proteins involved with transport such as importin and exportin. To test whether acetylation could possibly affect Smad2 localization, immunofluorescence experiments were conducted in Hep3B cells. Initially, in experiments where a fairly high amount of Smad2 or Smad2 2K19R was being overexpressed, we did not observe any major difference in the localization of Smad2. However, when the levels of expressed Smad2 or Smad2 2K19R were decreased and the cells were imaged using immunofluorescence viewed through confocal microscopy we were able to see a slight change in the localization of the protein. The Smad2 2K19R mutant seemed to exist in the cytoplasmic fraction of the cell more when compared to the Smad2 wild type in TGF-ß stimulated cells. In the cells unstimulated state, Smad2 normally shuttles in and out of the nucleus. However, upon TGF-ß stimulation the Smad2 is retained in the nuclear fraction allowing more transcription of TGF-ß target genes. When TGF-ß signaling ceases it is not entirely clear what happens to the Smad2. Some authors claim degradation of the Smad complex occurs while others claim the Smad2 becomes dephosphorylated and shuttles back to the cytoplasm. Most likely, a combination of the two occurs to different levels in different cell types. Because acetylation most likely occurs in the nucleus where p300, CBP, and other acetylases reside, we believe that the increase of cytoplasmic Smad2 2K19R after TGF-ß stimulation indicates a loss of nuclear retention and an increase of nuclear export back into the cytoplasm. To test this hypothesis, we will be conducting experiments to test whether using nuclear export inhibitors will decrease the amount of Smad2 2K19R found in the cytoplasm, test the ability of Smad2 2K19R to interact with export and import factors, and also use a timed GFP tag to determine if the cytoplasmic Smad2 2K19R we observe is newly synthesized or not indicating whether it has been continuously shuttling in and out of the nucleus.

Because many could argue that what we are seeing is a consequence of over-expressed Smad2, we are extremely in creating stable cell lines expressing wild type Smad2 and Smad2 2K19R. Concurrently, we are also making stable cell lines with siRNA for Smad2 to reduce the amount of endogenous Smad2. Afterwards, Smad2 and Smad2 2K19R will be re-introduced into the si cell lines to see if a stable cell line expressing Smad2 or Smad2 2K19R close to endogenous levels can be generated. Then we were be able to repeat many experiments in a more "physiologically relevant" setting.

Once we have determined the consequence of Smad2 acetylation we will have a better idea of how this post-translational modification plays a role in the regulation of the TGF-ß signaling pathway. This knowledge will perhaps provide new insight into how the TGF-ß

signaling pathway can play a role in cancer initiation and progression, how this modification may also regulate other pathways relevant to cancer, and may suggest new ideas as to how to treat or diagnose breast cancer.

Key Research Accomplishments

- Mapped residues required for acetylation and made non-acetylated Smad2 mutant
- Determined GAG region required for Smad2 acetylation to occur
- Determined Smad2 acetylation does not affect Smad2 phosphorylation, binding to Smad4 and Fast1, and DNA binding ability.
- Data suggests Smad2 acetylation does not affect protein stability, but requires final confirmation
- Data suggests Smad2 acetylation may alter Smad2 subcellular localization, possibly by loss of nuclear retention through increased protein export

Reportable Outcomes

- Presented data at the DOD Era of Hope 2004 conference in Philadelphia – June 2004.

Conclusions

- Smad2 acetylation requires lysines 19 and 20
- Smad2 acetylation requires presence of GAG region
- Smad2 acetylation does not alter Smad2 phosphorylation, binding to Smad4 and Fast1, and DNA binding ability.
- Data suggests Smad2 acetylation does not affect protein stability, but requires final confirmation
- Data suggests Smad2 acetylation may alter Smad2 subcellular localization, possibly by loss of nuclear retention through increased protein export

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Appendix
Abstract from Era of Hope 2005 Conference

SMAD2 ACETYLATION IN THE TGF-β SIGNALING PATHWAY

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Cells have developed complex mechanisms to prevent inappropriate cell division from occurring. These precautions are important for the prevention of tumor growth and loss of these mechanisms can lead to tumor progression. One pathway used to control cell growth is the transforming growth factor-β (TGF-β) pathway. Initiation of the TGF-β signaling pathway results in a wide range of biological functions including cell growth arrest. Loss of regulation of the TGF-β signaling pathways has been observed in breast cancer and 50% of all pancreatic cancer. Therefore, understanding how TGF-β is regulated is essential in our understanding of how cancer arises and progresses.

The TGF-β signaling pathway is mediated primarily through the intracellular Smad proteins. Many cancer cells have displayed either decreased levels or mutations in the Smad proteins, indicating an important role in the prevention of cancer progression. Smad proteins are regulated at several stages of TGF-β signaling through protein modifications such as phosphorylation and interactions with other proteins.

Recently, the receptor regulated Smad2 was identified as an acetylated protein through in vitro acetylation assays. Acetylation, a relatively new post-translations modification, has been shown to affect protein stability, localization, and DNA and protein binding ability. The acetylation of Smad2 was further confirmed by co-transfecting different Smad proteins in the presence of p300 into cells and performing a Western Blot using an antibody specific to acetylated lysines (Fig 1). Interestingly, Smad3, another receptor regulated Smad with 92% sequence identity to Smad2, cannot be acetylated.

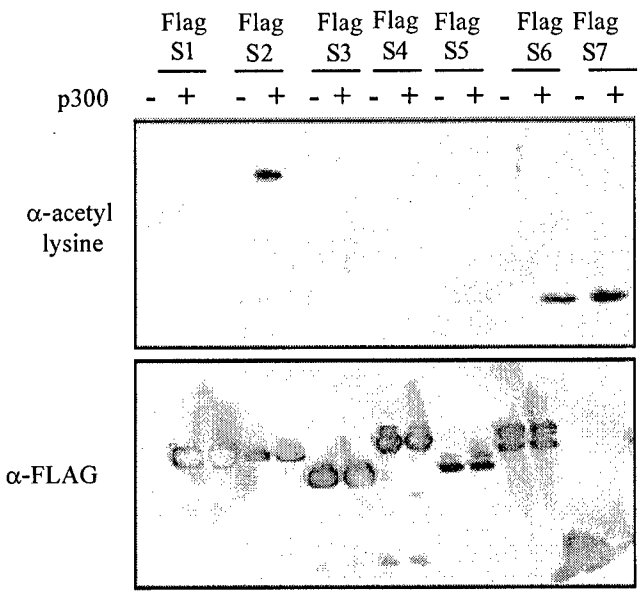
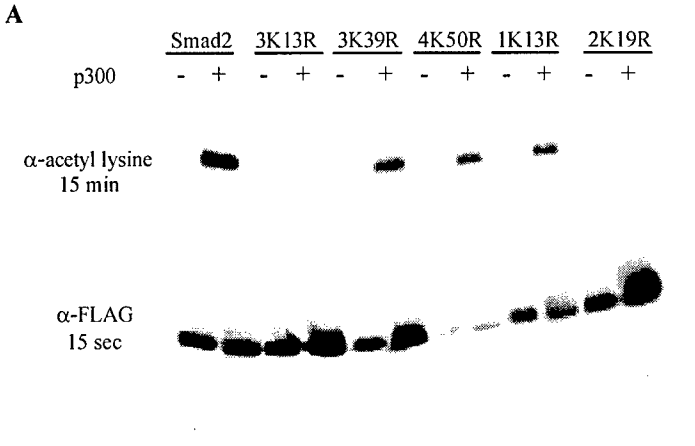


Fig1: Fl-Smad proteins transfected into 293T cells. Cells were lysed and FLAG proteins immunoprecipitated. The product was run on SDS-PAGE gel and subjected to Western blots with the indicated antibodies

A series of Smad1/Smad2 chimeras indicated that acetylation of Smad2 occurs in the MH1 domain of Smad2. Swapping the MH1 domain of Smad2 and Smad3 confirms that acetylation occurs in the MH1 domain. Point mutations of lysines in the MH1 domain identified lysines 19 and 20 as

residues required for Smad2 acetylation (Fig. 2). Performing mass spec on acetylated Smad2 will test whether lysines 19/20 are the actual sites of acetylation or if they are merely regulatory.



B

	13	19 20	39	43	46	50 51	53 54
Smad2 3K13R	**R**	**RR**	K	K	K	KK	KK
Smad2 3K43R	K	KK	**R**	**R**	**R**	KK	KK
Smad2 4K50R	K	KK	K	K	K	**RR**	**RR**
Smad2 1K13R	**R**	KK	K	K	K	KK	KK
Smad2 2K19R	K	**RR**	K	K	K	KK	KK

Fig2: Acetylation of Smad2 pt. mutants
A. 293T cells were cotransfected with Smad2 point mutants +/- p300. Cells were lysed and Flag protein was immunoprecipitated The protein was then subjected to Western Blot with the indicated antibodies.
B. Schematic showing which lysines were mutated to arginines.

Functional tests to determine the consequences of Smad2 acetylation will be conclusively performed. Preliminary results suggest that a loss of Smad2 acetylation does not play a role in the protein's stability nor does it alter its phosphorylation state. Other potential factors affected by acetylation to be checked include protein-protein interactions, protein-DNA interactions, and localization of the protein. Ascertaining the role of Smad2 acetylation in the TGF-β pathway gives us further insight as to how abrogations in the TGF-β pathway can lead to initiation and progression of breast cancer. It may also lend insight into how other pathways implicated in breast cancer are regulated. All this information only increases our knowledge of breast cancer and will ultimately lead to better forms of prevention, detection, and treatment of this disease.

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